Interferon Test Procedures:
Points to Consider in the Production and Testing of Interferon Intended for Investigational Use in Humans

Office of Biologics
National Center for Drugs and Biologics

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I. Introduction

We previously distributed a document entitled, "Interferon Test Procedures" (July 1980, revised February 1, 1981), which was intended to represent areas of general agreement of participants at a workshop held at the NIH on October 29, 1979. Areas of discussion focused on safety, purity, potency, and tests of clinical correlates. The resulting suggestions regarding production and testing of interferon (IFN) materials were intended to facilitate progress in the development of IFN products for investigational use. It was anticipated that new developments in the manufacture of IFN would require re-evaluation of those suggestions.

This, in fact, has been the case. The application of new technologies has resulted in better defined products with higher specific activities; additional preparations of all IFN types are becoming more readily available for clinical trials; expanding clinical studies are resulting in the accumulation of clinical data which can now be subjected to further analysis. It has become apparent that the previous "Interferon Test Procedures" are no longer appropriate. For example, provisions were made for certain bulk and final container tests using reduced volumes. This was considered reasonable since there was a desire to conserve material needed for clinical investigation. It was not intended, however, that these efforts to conserve material would be valid or appropriate as lot sizes increased and material became more readily available. However, in those circumstances where the availability of specific IFN preparations is limited, we will consider reduced volume testing on a case-by-case basis.

We believe it is timely to reconsider previous suggestions in the light of these new data and to explore further those questions which were, due to lack of experience, difficult to address. As with the previous document, the points discussed below should be interpreted as being what the Office of Biologics (OB) generally expects manufacturers of IFN to consider during drug development. These points are not all-inclusive, and certain of them may not be applicable to all situations. Therefore, the OB will review the adequacy of testing of any product on a case-by-case basis.

Many aspects of manufacturing and of the production facility are not discussed here. Such specific details and the related concerns of interested manufacturers should more properly be discussed directly with the OB and should be included in specific IND and product license applications. In general, the Current Good Manufacturing Practice regulations (GMP's) and the biologics GMP's pertinent to manufacturing and control testing (21 CFR 210, 211 and 600 et seq.) are applicable.

We hope that this guidance will prove useful to those interested in organizing a filing for submission to the Agency.

II. Manufacturing Process

A. Characterization of Cell Substrate for IFN Production

The objective of characterization of cell substrates is to assure that consistent, well characterized lots of IFN can be produced from a standardized substrate to provide assurance of product consistency and safety. The type of testing needed, therefore, depends on the nature of the substrate used.

1. Source: Leukocytes (Buffy Coats)

Procedures for collection and testing of source leukocytes pertain to donor qualifications, methods for blood collection, processing, storage, and hepatitis B virus antigen testing. Guidelines for the Collection of Human Leukocytes for Further Manufacturing were distributed in January 1981, and copies of these guidelines are available on request (Dockets Management Branch, HFA-305, FDA, Room 4-62, 5600 Fishers Lane, Rockville, MD 20857).

2. Source: Human Diploid Cells

Human diploid fibroblast cell lines used for the production of IFN should have a well-defined passage history and be demonstrated to be stable and diploid (Report of Ad Hoc Committee on Karyological Control of Human Cell Substrates, J. Biol. Standardization 1979, 7 397-404). A cell bank should be established from which aliquots are used to establish production cultures. The cell bank must be shown to be free from adventitious agents e.g., bacteria, fungi, mycoplasma (see attachment), and viruses. In addition, the cell banks should be shown to be non-tumorigenic in a suitable animal model. Also, a portion of the cell cultures used for the production of each lot of IFN should be retained as control cultures and tested for identity and the absence of adventitious agents.

Information from studies on continuous cell lines and products derived from them is needed to resolve evolving issues pertaining to use of such cell lines. Until such information is available, much will be learned from testing continuous cell line substrates for their adequacy, consistency and safety for use. These tests should be directed toward providing information that will serve as a basis for establishing scientific consensus on the safety and efficacy of cell culture derived biological products.

A cell bank should be established and tested for adventitious agents as described in II., A., 2. Important information may be obtained from testing the cell bank of the continuous cell line for tumorigenic potential. Karyotype and stability of the cell bank should also be determined and these tests repeated periodically during production.

4. Cells Used to Produce Recombinant DNA-Derived Products

The biologic regulations in 21 CFR 610.18 prescribe requirements for storing, maintaining, identifying, and verifying cultures of bacteria or yeast. Continuous cell lines should be described and characterized as in II., A., 3. In addition, the following information would provide a data base for future IFN preparations and may assist in solving potential problems, e.g., an inactive IFN preparation resulting from a deletion or insertion in the nucleotide sequence:

a. the nucleotide sequence of the cloned human IFN gene(s) present in the seed stocks used for production runs;

b. the characterization of the vector, e.g., restriction enzyme mapping, drug resistance markers, control of copy number, etc.;

B. Inducers and Purification/Characterization

The following information regarding inducers and purification/characterization should be submitted.

1. Inducers

a. A description of the biological and/or chemical nature of the inducer, e.g., structure, solubility, contaminants, by-products, stability.

b. Adverse properties of the inducers, e.g., toxicity, carcinogenicity, pyrogenicity.

c. A description of the procedures used to remove and assay for residual inducers.

d. Specifications for residual levels of inducers in the final products.

2. Purification/Characterization

a. Information demonstrating that the purification procedure for IFN preparations adequately removes unwanted material from the final product. Whenever possible, confirmatory testing should demonstrate that deliberately added contaminants are effectively removed from the final product by the purification procedure.

b. If affinity chromatography is used, data relating to the identity, purity, and safety of the ligand and the amount of contaminating ligand in the final product; and a description of procedures for column preparation, maintenance, preelution, elution and storage, as well as criteria indicating the need for column replacement.

c. If IFN is derived from continuous cell lines, results of tests for residual DNA. In the absence of defined tests for measuring residual biologically active DNA, information relating to elimination of DNA by the manufacturing method to the extent that current technology allows detection (approximately 10 pg DNA by hybridization analysis). It should be noted that tests for biologically active DNA e.g., assays for the presence of specific oncogenes are being evaluated.

d. Information relating to the inactivation and kinetics of virus when used as an inducer during the manufacturing process.

e. 21 CFR 610.15(b) and (c) prescribe requirements for the presence of foreign proteins and/or antibiotics. The acceptability of antibiotics other than penicillin and their concentrations in the product(s) will be evaluated on a case by case basis.

f. IFN preparations used in clinical studies should be as pure as possible. In general, the specific activities of available partially purified HuIFN-alpha and HuIFN-beta are $>10^6$ units/mg protein and that of HuIFN-gamma are $>10^5$ units/mg protein.

In some cases IFN preparations with higher specific activities are desirable depending on the origin of the cell line, inducer, and the clinical study design. In each case the material produced should exhibit a consistent specific activity and, where applicable, contaminants should be identified.

The characteristics of an IFN preparation should include information on its antigenicity; solubility (non-sedimentable at 100,000 xg for 2 hours); protease susceptibility, i.e., loss of antiviral activity following protease treatment; pH2 stability; mobility on SDS PAGE (protein staining and eluted antiviral activity pattern); examination of its interaction with appropriate monoclonal or polyclonal antibodies; behavior on high performance liquid chromatography (HPLC); contamination with other biological activities, e.g., lymphokines (interleukin I, II), endogeneous pyrogens, proteases; stability of bioactivity as a function of time and temperature; primary amino acid sequence; post translational modification; and state of aggregation/degradation. Altered forms, if they cannot be removed, should be further characterized when feasible, with regard to molecular weight, antiviral specific activity, and pyrogenicity.

g. IFN products derived from recombinant DNA technology are likely to contain contaminants which are not found in natural IFN preparations. Therefore, in addition to the previously described comments, the following procedures should be considered when determining product purity for each lot of recombinant DNA-derived human IFN:

(1) Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). SDS PAGE could be used to assess product purity and estimate the apparent molecular weight of the protein(s) or peptide(s) in the preparation. The gels should be run in the presence and absence of reducing agents and with molecular weight standards. Staining with both Coomassie brilliant blue and silver is recommended. Isoelectric focusing or two dimensional gel electrophoresis can be applied to evaluate product molecular heterogeneity and the presence of some bacterial components. Contaminants in the preparation should be identified by radioimmunoassay or western blotting. In general, >95% of the protein should be the expected IFN species.

IFN aggregates or degradation products should be identified and removed if found biologically distinct from the parent molecule. If biologically identical to the parent molecule, they should be present in consistent amounts in each preparation.

(2) Specific activity. The IFN should be purified to a constant and consistent specific activity. In general, the specific activity of recombinant IFN's have been in the order of 10^8 reference units per mg protein. NIH or other accepted international human IFN reference standards should be used.

(3) Amino Acid Composition. The amino acid composition should be determined and be consistent with the gene sequence and/or published composition for the native IFN. It should display the absence of muramic acid, or any other components indicative of bacteria or yeast.

(4) Partial Sequence Analysis. Partial amino-terminal sequence analysis (for example, 15 residues) and carboxy-terminal sequence analysis could serve as important criteria of purity for R-DNA produced proteins or peptides. The sequence data presented in tabular form should include both the total yield and the repetitive yield for each amino acid at each cycle. Unexpected heterogeneity in the amino terminus and carboxy terminus due to proteolytic degradation of proteins produced by R-DNA technology have been revealed by such sequence analysis.

(5) High Performance Liquid Chromatography. This method can be used to assess protein purity. Greater than or equal to 95% of the protein should elute as a single peak using reverse phase chromatography in each of two different solvent systems.

(6) Peptide Mapping. Peptide mapping is a useful criterion for comparing a recombinant IFN to its native counterpart IFN or retention samples. In addition, it serves to identify the IFN preparation. It is an important

method for the assessment of correct disulfide bond formation. This can be accomplished using specific proteases in combination with high performance liquid chromatography or two dimensional electrophoresis.

All of the above procedures (g., 1. - 6.), need not be conducted for each lot; however, whenever there is a significant change in protocol or in preparative material used, e.g., when a new lot of column material is introduced, the purification procedure should be re-evaluated for its adequacy to remove contaminants.

C. Consistency of Manufacture

Biological products derived from living cells are subject to variabilities inherent in complex biological systems. Product consistency should, therefore, be maintained by close attention to consistency of manufacturing procedures as well as analysis of the final product. Inherent in this concept is the need to understand what important variables are involved and how they may be affected during manufacture. For example, multiple species of IFN are likely to be present in products derived from eukaryotic cells and may be present in IFNs derived from bacteria. Other biologically-active substances with important clinical effects such as lymphokines, cytokines, or endotoxin may also be present. Cellular enzymes may be present which affect the physical state of IFN in the final product, such as

by formation of oligomers of IFN. Manufacturers should establish rigorous quality control procedures to detect and reduce product variability.

Manufacturers should consider the methods used for production and purification of their individual products to judge which additional biologic activities are potentially relevant to their product and should be evaluated.

III. Bulk Lot Tests

For the purposes of the following suggestions, the term "bulk lot" is defined as the contents of a single container (B) derived from single or multiple harvests which is subject to further manufacturing procedures. If a preliminary bulk is prepared and subdivided into several separate containers (B_1, B_2, etc.) from which final containers (FC) are filled, the contents of each of those containers (B_1, B_2, etc.) is considered to be a separate bulk for testing purposes and each should bear a distinguishing lot number.

Tests for mycoplasma should be performed on preclarified bulk materials for products derived from lymphoblastoid, fibroblast, or continuous cell lines (see attachment). Alternately, tests for mycoplasma may be performed at any other point in the manufacturing process provided the sampling adequately represents the material comprising a bulk lot and represents a point beyond which mycoplasma reproduction is not feasible. Manufacturers may wish to propose alternate tests for mycoplasma.

Preclarified bulk IFN derived from cells in culture should be tested for sterility prior to further processing. Final bulk sterility testing is prescribed in 21 CFR 610.12.

If a protein is added to stabilize an IFN preparation, the specific activity of the bulk lot prior to the addition of the exogenous protein should be determined.

IV. Final Container Tests

The definition of and testing procedures for final containers are as follows:

A. Definition of final container lot: that group of final containers, identical in all respects, which have been filled from a single bulk lot container (B) without any changes that will affect the integrity of the filling assembly. For testing purposes, if a filling is subdivided among more than one freeze-drying operation, each such operation will be considered to be a different final container lot and each should bear a distinguishing lot number.

B. Pyrogenicity: the importance of pyrogenicity testing of the final container material has not yet been ascertained. Many preparations of highly purified IFNs, both native and recombinant, exhibit very low endotoxin levels, as determined by the limulus amebocyte lysate

(LAL) test, and yet elicit pyrogenic responses in humans and rabbits. It is at present uncertain whether pyrogenicity is inherent to IFNs, or attributable to a nonendotoxin contaminant presently undetected, or whether other factors are involved. We therefore recommend that both the rabbit pyrogen and LAL tests be performed on each lot. Rabbit tests must be performed using a dose (antiviral units/kg of body weight) equivalent to that anticipated to be the maximum human dose as required in 21 CFR 610.13. Volumes not less than 1 ml/kg and not greater than 10 ml/kg should be used in the rabbit pyrogen test. If samples are to be diluted, nonpyrogenic sterile saline should be used. If a positive rabbit test is observed with this dose, it is recommended that serial 0.5 $\log_{10}$ solutions of the final container material be tested in order to determine the level of IFN activity which will give negative results. A positive rabbit test, however, will not necessarily of itself be a basis for rejection; the OB will review the acceptability of pyrogenic material on a case-by-case basis. Limulus testing should be carried out as recommended by the lysate manufacturer and the test should include controls designed to show that the IFN preparation being tested does not impair the sensitivity of the test. The patient population, the degree of pyrogenicity of the IFN, and the route of its administration should all be considered in the assessment of the acceptability of any given lot of IFN (Docket No. 79D-0465 HUMAN, BIOLOGICAL, AND ANIMAL DRUGS AND MEDICAL DEVICES; AVAILABILITY OF DRAFT GUIDELINE FOR USE OF THE LIMILUS AMEBOCYTE LYSATE (LAL) TEST).

C. The final container general safety test (21 CFR 610.11), performed to detect extraneous toxic contaminants, is applicable to all IFN

preparations. Exceptions may be made for specific topical preparations after consultation with the OB. These exceptions would only be considered if a more appropriate test is available.

D. Potency testing of IFNs: virus interference assays are the procedures most commonly used to measure potency of IFNs. Biologic activities of IFNs are also reflected in other laboratory procedures which measure immuno-enhancing and antiproliferative effects, but standard methods for performing these assays are not generally available. Assays which measure IFN content such as protein determinations and radio- and enzyme-linked immunoassays have also been established in some laboratories, but the extent to which results from these tests correlate with biologic activity needs to be established on an individual basis. Therefore, potency of IFNs should continue to be tested in virus interference assays. The most appropriate method for performing these tests will undergo periodic re-evaluation as more information becomes available. At present, the following considerations should be taken into account:

1. Cell lines: a variety of cell lines are sensitive to IFN effects when challenged with viruses; and cell lines of non-human origin may be more sensitive to effects of some IFNs than human cell lines. Unless there is evidence that clinical effects of a given IFN preparation are better correlated with potency determined in a non-human cell line, then the use of human cell lines is more desirable. In testing IFNs for which reference reagents exist, cell lines should be selected which are sensitive to the reference at the assigned potency. In the case

of gamma IFNs, the same principle may apply when a reference becomes available. In the absence of availability of a standard reference, gamma IFNs should be tested on a sensitive, well characterized cell line and compared to a working reference standard.

2. Viruses: vesicular stomatitis virus, encephalomyocarditis virus, and sindbis virus are potentially suitable for determination of IFN potency. However, whichever virus is used, standardization of the amount of virus used is essential. The extent to which variability of virus inoculum affects variability of the assay results should be determined and should be the basis for establishing limits of acceptability of individual tests. A titration of the virus inoculum used should be performed in each test to determine acceptability of results.

3. Reference standards: laboratory working standards should be established. For alpha (leukocyte, lymphoblastoid cell, or recombinant DNA-derived) and beta (fibroblast and recombinant DNA-derived) IFNs, internal laboratory standards should be characterized in comparison to generally available reference standards such as those provided by the NIH. Until a gamma IFN reference reagent becomes generally available, each laboratory should establish an internal standard for assay of potency. The variability of results obtained for standards should be evaluated and limits of acceptability of individual test results

established. Potency results for IFNs under test should be determined by comparison to results obtained for the standard in the individual tests.

4. Establishment of confidence intervals for test results: well defined potency test procedures should be evaluated for consistency. The number of replicates required in individual tests and the number of repeat tests required to give adequate assurance of consistency should be established. Confidence intervals should be determined for each final potency result and should reflect reasonable consistency of the assay.

In addition to testing IFNs for potency in virus interference assays, manufacturers should consider the relevance of tests of other biologic activities to potential mechanisms of action in clinical studies. Any testing which would facilitate understanding of clinical effects is encouraged. Immunoassays of IFN potency offer the potential advantages of improved ease of performance, sensitivity, and consistency. However, the suitability of such assays for routine potency testing must be established individually in any laboratory desiring to do so, by demonstration that potency results obtained through immunoassays consistently correlate with biological activity under a variety of circumstances. The types of information needed to establish this correlation should be determined through consultation with the OB.

Test for Mycoplasma in Interferon Bulk Material
(Lymphoblastoid, Fibroblast, or Other Cell Line Produced Material)

Prior to clarification (or filtration), each lot shall be tested for the presence of mycoplasmas, as follows: samples of the bulk lot shall be stored before use either: a) between 2°C and 8°C for no longer than 24 hours; or b) at -20°C or lower if stored for longer than 24 hours. The bulk lot shall be tested for the presence of mycoplasmas by using an agar and broth procedure. The media shall be such as have been shown to be capable of detecting known Mycoplasmas and each test shall include control cultures of at least two known strains of Mycoplasma, one of which must be M. pneumoniae. Each lot of agar and broth media used shall be examined for growth-promoting properties and shown to be capable of detecting mycoplasma contamination as described below.

No less than 0.4 ml of the bulk lot shall be inoculated, in evenly distributed amounts, over the surface of four or more plates of agar media; and no less than 1 ml of the bulk lot shall be inoculated into 25 ml of broth medium. After 3 and 14 days of incubation, 0.4 ml of the broth cultures shall be tested by subculture onto four or more agar plates. One-half of the initial isolation plates and one-half of the subculture plates shall be incubated aerobically in containers designed to prevent dessication, and the remaining culture plates incubated in a 5 to 10% carbon dioxide in nitrogen atmosphere. Agar and broth cultures shall be incubated at 35 $\pm$ 1°C.

All inoculated plates shall be incubated for no less than 14 days and observed for growth of mycoplasma colonies microscopically at no less than 300 times magnification. If the Dienes (methylene blue-azure dye) or an equivalent staining procedure is used to examine plates for colony growth, at least one square cm plug of the agar must be examined. The presence of the Mycoplasma shall be determined by comparison of the growth obtained from the test samples with that of the control cultures, with respect to typical colonial and microscopic morphology. The bulk lot is satisfactory if none of the tests on the samples show evidence of the presence of Mycoplasma.